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Kinetic Characterization of Spinach Leaf Sucrose-Phosphate Synthase[1]

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ABSTRACT

The spinach (*Spinacia oleracea*) leaf sucrose-phosphate synthase was partially purified via DEAE-cellulose chromatography, and its kinetic properties were studied. Fructose-6-phosphate saturation curves were sigmoidal, while UDPglucose saturation curves were hyperbolic. At subsaturating concentrations of fructose-6-phosphate, 1,5 anhydroglucitol-6-phosphate had a stimulatory effect on enzyme activity, suggesting multiple and interacting fructose-6-phosphate sites on sucrose-phosphate synthase. The concentrations required for 50% of maximal activity were 3.0 millimolar and 1.3 millimolar, respectively, for fructose-6-phosphate and UDPglucose. The enzyme was not stimulated by divalent cations. Inorganic phosphate proved to be a potent inhibitor, particularly at low concentrations of substrate. Phosphate inhibition was competitive with UDPglucose, and its K_i was determined to be 1.75 millimolar. Sucrose phosphate, the product of the reaction, was also shown to be a competitive inhibitor towards UDPglucose concentration and had K_i of 0.4 millimolar. The kinetic results suggest that spinach leaf sucrose-phospahte synthase is a regulatory enzyme and that its activity is modulated by the concentrations of phosphate, fructose-6-phosphate, and UDPglucose occurring in the cytoplasm of the leaf cell.

The biosynthesis of sucrose-P was first reported by Leloir and Cardini (14) to occur via transfer of glucose from UDPglucose to fructose-6-P. Hawker and Hatch (10) and Hawker (7) subsequently reported the presence in plant tissues of a specific sucrose-6-P phosphatase. Earlier labeling studies (1, 3, 4) suggested that, in leaves, sucrose was synthesized from hexose phosphates and not from free sugars in the cell, and that implicated sucrose-P synthase as the enzyme responsible for sucrose synthesis in plants.

The enzyme isolated from wheat germ has been highly purified and studied in great detail (18, 23). Some of its kinetic properties have been postulated to be pertinent to its regulation *in vivo* (19, 23). However, very little has been published on the kinetic properties of the leaf sucrose-P synthase, even though the enzyme activity has been detected in various leaves (2, 6, 9, 13, 16, 17, 20).

The following describes a partial purification of the spinach leaf sucrose-P synthase and its preliminary kinetic characterization. Emphasis of the studies were directed to determining possible regulatory modes of the enzymic activity.

MATERIALS AND METHODS

Reagents. UDP-[^{14}C]glucose was obtained from New England Nuclear Co. *Escherichia coli* alkaline phosphatase ($[NH_4]_2SO_4$ fraction) and insoluble PVP were obtained from Sigma Chemical Co. DEAE cellulose (DE-52) was obtained from Whatman. All other reagents were obtained at the highest purity available.

Plant Material. Spinach (*Spinacia oleracea*) was bought from a supermarket.

Preparation of the Enzyme. All operations, unless otherwise specified, were carried out at 4°C. One hundred g of washed and deveined spinach leaves were frozen with liquid N_2 and ground in a mortar with a pestle. The frozen powder was added to a slurry of 50 g insoluble PVP (Sigma P-6755) in 200 ml extraction solution. The extraction solution contained the following: 100 mM Hepes (pH 7.5); 20 mM β-mercaptoethanol; 2 mM EDTA and 2% ethyleneglycol. The extraction was carried out in a vacuum flask under an atmosphere of N_2. After the extract had been squeezed through a cheese cloth, it was centrifuged at 16,000*g* for 20 min, and the supernatant fluid was saved. This solution was poured onto a DEAE-cellulose column (2.5 × 20 cm), previously equilibrated with a buffer solution, containing 20 mM Hepes-NaOH (pH 7.5), 10% ethyleneglycol, 2 mM Na_2EDTA, and 5 mM β-mercaptoethanol. The column was washed with about 200 ml of the above buffer, and the enzyme was eluted with a 500 ml linear NaCl gradient (0 to 0.5 M) in the same buffer. The fractions with the sucrose-P synthase activity were pooled and concentrated in an Amicon TCF 10 thin-channel cell with a PM-10 membrane. After concentration to about 5 ml, the enzyme fraction was diluted to 20 ml with the above DEAE cellulose equilibration buffer. The enzyme was frozen and stored in liquid N_2.

Enzyme Assays.

Assay of Sucrose-P Synthase. The reaction mixture contained the following: 5 μmol Hepes-NaOH (pH 7.5); 1.2 μmol fructose-6-P; 1.2 μmol UDP-[^{14}C]glucose (specific activity, 100,000 to 400,000 cpm/μmol); 0.2 mg BSA; 0.5 μmol $MgCl_2$; and enzyme; in a total volume of 0.125 ml. After 20 min at 37°C, the reaction was stopped by immersing the tubes in boiling water for 1 min. The sucrose-6-P formed was hydrolyzed to sucrose with *E. coli* alkaline phosphatase (0.1 unit/assay). After incubation for 30 min at 37°C, 0.4 ml of a 5% sucrose solution was added. The diluted reaction mixture was loaded onto a 1-ml DEAE cellulose column (DE-52) and eluted with 5 ml H_2O (pH 7.5). A 1-ml sample was added to 5 ml of phase-combining system for aqueous sample (Amersham Co.), and the radioactivity was determined in a liquid scintillation counter. No enzyme control values were 100 to 250 cpm. A similar assay procedure utilizing alkaline phosphatase has been reported by Salerno *et al.* (24).

One unit of enzyme activity is defined as 1 μmol of sucrose or sucrose-6-P produced in 10 min, under the conditions described above. The production of sucrose-6-P was linear for 60 min. In all subsequent experiments, rate-limiting quantities of enzyme were used, and the amount of activity was measured during the first 20 min. Under such conditions, the rate of enzyme increased linearly with protein concentration.

Assay of Sucrose-P Phosphatase Activity. By using the above

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assay and omitting the hydrolysis step of sucrose-6-P by alkaline phosphatase, it is possible to determine how much of the sucrose-6-P formed was converted to sucrose by phosphatase action. The released sucrose was measured as in the sucrose-phosphate synthase assay.

Assay of Sucrose Synthetase. The reaction mixture is the same as that for the sucrose-P synthetase assay, except that 10 mM fructose replaced fructose-6-P.

Protein Determination. Protein was determined according to the method of Lowry *et al.* (15).

Chromatography. The product of the reaction sucrose-6-P was identified by descending paper chromatography on Whatman No. 1 paper, using the following three solvents: 95% ethanol, 1 M ammonium acetate (pH 7.5) (5:2 v/v); 1-butanol, pyridine, H_2O (6:4:3); 1-butanol-acetic acid-H_2O (4:1:5). The radioactivity in the spots was measured with a Nuclear Chicago strip counter. Sugars were detected with $AgNO_3$-NaOH (25).

RESULTS

Enzyme Purification and Stability. Two important requirements are necessary to keep the enzyme active during purification: namely, high concentration of reducing agent (20 mM β-mercaptoethanol); and the presence of a phenol absorbing agent, such as PVP. The best purification results were achieved by performing the extraction under N_2 atmosphere. Some representative data of enzyme purification are shown in Table I.

A typical chromatographic pattern on DEAE-cellulose and the activities of sucrose-P phosphatase, sucrose-P synthase, and sucrose synthase are presented in Figure 1. A small amount of sucrose-P phosphatase and sucrose synthase activity were seen with the sucrose-P synthase fractions. However, the sucrose synthase activity in the preparation disappeared completely after freezing in liquid N_2. Phosphohexose isomerase activity in the

Table I. *Purification of Spinach Sucrose-P Synthase*

These results are based on 100 g fresh weight of leaves.

Purification Step	Volume	Protein	Total Activity	Specific Activity
	ml	*mg*	*units*	*units/mg*
Crude extract	375	555.0	72.1	0.13
DEAE-cellulose	20	28.2	35.7	1.27

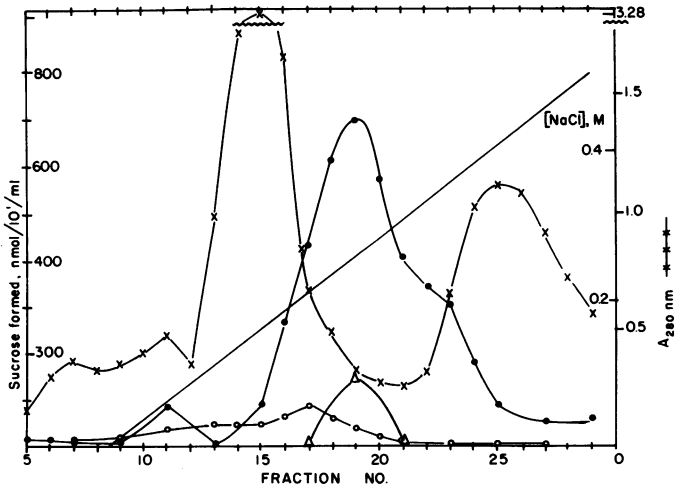

FIG. 1. DEAE cellulose chromatography of sucrose-P synthase. The chromatography procedure and assay methods are described under "Materials and Methods." ×——×, A_{280} nm; ●——●, sucrose-P synthase activity; ○——○, sucrose synthase activity; and △——△, sucrose-P phosphatase activity.

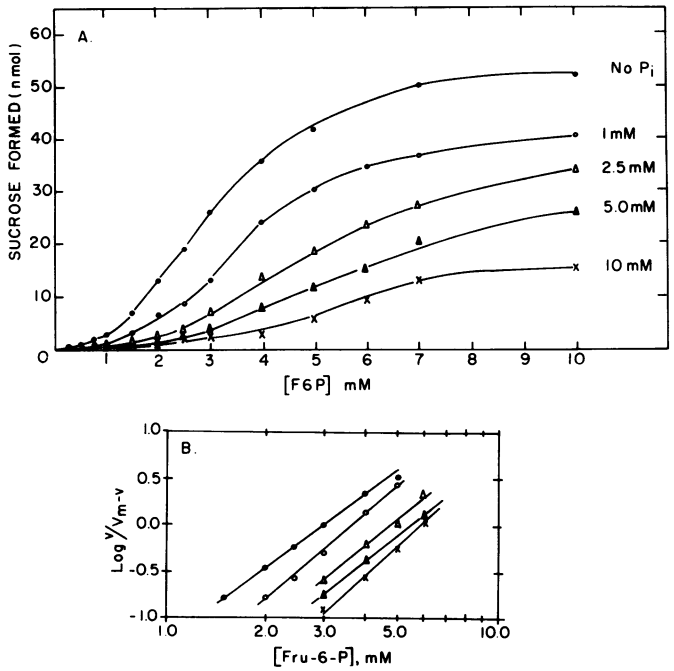

FIG. 2. Effect of fructose-6-P (F6P) concentration on sucrose-P synthase activity in the absence and presence of Pi. The concentration of Pi is indicated. B, Hill plot of the data. The conditions of the assay are indicated in "Materials and Methods."

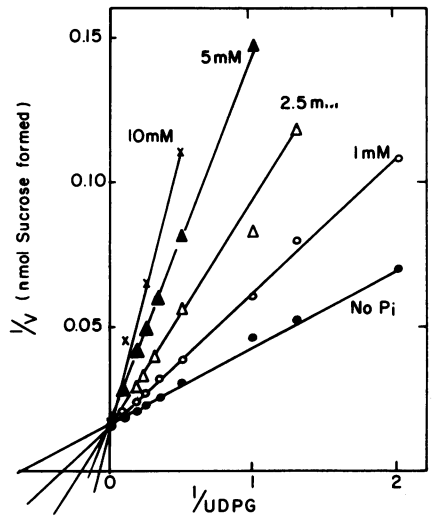

FIG. 3. Reciprocal plot of rate of sucrose formation *versus* UDPglucose concentration in the absence and presence of Pi. The concentrations of Pi used are indicated.

DEAE fraction was present at a level of 0.4 μmol/min · mg protein. No UDPglucose-degrading activity nor UDPgalactose epimerase activity was found to be present in the DEAE fraction. When the enzyme solution was frozen and kept in liquid N_2, sucrose-P synthase activity remained stable for at least 11 months. The enzyme lost 50% of its activity in 4 weeks at 0°C.

Identification of Reaction Product. The product of reaction was characterized by paper chromatogrpahy using three solvent systems. In solvent A, the compound produced when fructose-6-P and UDP-[^{14}C]glucose were used as substrates cochromatographed with a sucrose-P standard. Very little radioactivity (about 5 to 10%) was associated with the sucrose standard. The standard sucrose-6-P used was prepared as essentially described with the wheat germ enzyme (14). When the product was incubated with

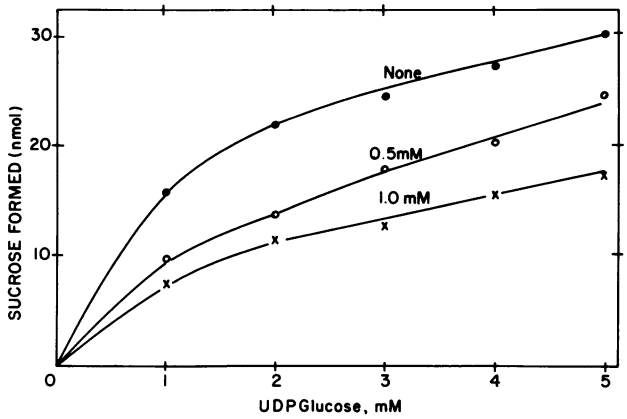

FIG. 4. Inhibition of sucrose-P synthase activity by sucrose-P. The concentrations of sucrose-P are indicated. The assay conditions and procedures are indicated in "Materials and Methods."

Table II. *Effect of Various Compounds on Sucrose-P Synthase Activity*
All the compounds were at 1 mM concentration.

	Sucrose Formed	
	Fructose-6-P, 2 mM	Fructose-6-P, 3 mM
	nmol	
None	14.9	28.8
Pyruvate	15.2	30.4
P-Enolpyruvate	11.8	26.0
NAD	13.3	25.9
NADH	15.6	27.9
3-P-Glycerate	17.2	37.5
NADP	14.2	27.9
Sucrose	15.1	29.4
Sucrose-6-P	6.1	12.3
UDP	11.7	23.9
Fructose 1, 6-P_2	11.3	21.3
Ribose-5-P	16.2	29.1

Table III. *Effect of Fructose-6-P Analog on the Sucrose-P Synthase Activity*

	Sucrose Formed		
Fructose-6-P	1,5-Anhydro-glucitol-6-P, 0 mM	1,5-Anhydro-glucitol-6-P, 1.8 mM	1,5-Anhydro-glucitol-6-P, 3.2 mM
mM		*nmol*	
1.5	7.0	9.2	13.3
2.0	17	20	21.3
3.0	28	35	29
4.0	35	44	30

alkaline phosphatase, the only labeled compound detected coincided with the sucrose spot after chromatography.

pH Optimum. Sucrose-P synthase had its maximum activity in a broad pH range, from pH 6.5 to 7.5 in Hepes buffer. Tris-HCl buffer had a slight inhibitory effect (about 35%) on the enzyme activity.

Effect of Fructose-6-P Concentration. The saturation curve with fructose-6-P is presented in Figure 2. The shape of the curve is sigmoidal. Plotting the data according to the Hill equation yields a straight line with $S_{0.5}$ value (concentration of substrate giving 50% of maximal velocity) of 3.0 mM and Hill plot slope, $\bar{n}$, of 2.6. The sigmoidal saturation curve, with respect to fructose-6-P, was observed with enzyme preparations from leaves (17) and wheat germ (19), suggesting that its allosteric properties may be regulatory in the sucrose biosynthesis.

Effect of UDPGlucose Concentration. The saturation curve with UDPglucose was hyperbolic in shape. Double reciprocal plots curve indicated a K_m of 1.3 mM (Fig. 3).

Activation and Inhibition of the Enzyme. The effect of different compounds on sucrose-P synthase is shown in Table II. No significant stimulatory effect of different compounds could be observed. Sucrose-6-P was a potent inhibitor. The inhibitory effect of sucrose-6-P was studied in detail, and the results are shown in Figure 4. The inhibition toward UDPglucose concentration is competitive, and a K_i of 0.4 mM was obtained in reciprocal plots for sucrose-6-P. Sucrose up to a concentration of 100 mM had no effect on enzyme activity.

Effect of Cation and Anions. Divalent cations—Mg^{2+}, Mn^{2+}, and Ca^{2+}—up to a concentration of 10 mM did not affect enzyme activity. EDTA (20 mM) had no effect on enzyme activity. Pi, however, has a markedly inhibitory effect (Figs. 2 and 3). Pi at 10 mM increases the $S_{0.5}$ value for fructose-6-P from 3.0 mM to 5.9 mM. The Hill $\bar{n}$ slope values do not change dramatically, being 3.1, 2.9, 2.8, and 3.3 for inhibitor concentrations of 1.0, 2.5, 5.0, and 10 mM, respectively. The Pi inhibition is competitive toward UDPglucose, as can be seen from the double reciprocal plots of the data (Fig. 3). The K_i for Pi was calculated to be 1.75 mM.

Effect of Fructose-6-P Analogs. 2-Deoxyglucose-6-P at 4 mM concentration had no effect on the enzyme velocity at any fructose-6-P concentration tested. 1,5 Anhydroglucitol-6-P, however, had a markedly stimulatory effect on the enzyme activity at subsaturating concentrations of fructose-6-P (Table III). Both analogs, however, cannot act as substrate for the enzyme.

DISCUSSION

The above data indicate that the fructose-6-P saturation curve for the spinach leaf sucrose-P synthase is of sigmoidal shape. The results are, thus, similar to those obtained for the enzyme isolated from wheat germ (19), barley leaf, grape leaf, ladino clover leaf, sweet potato root, and potato tuber (17). These data may suggest multiple and interacting sites for fructose-6-P, and stimulation caused by the analog, 1,5 anhydroglucitol-6-P, that is inactive as a substrate is consistent with the concept of interacting sites.

Although the same type of saturation curve is observed with the wheat germ enzyme, other properties of the two enzymes appear to be different. The wheat germ enzyme is stimulated by Mg^{2+} (19, 23). However, the spinach leaf enzyme is not. Other sucrose-P synthases tested (17) either were not affected by Mg^{2+} or the stimulation was slight (10 to 25%). None of the leaf enzymes was stimulated by Mg^{2+}, nor did Mg^{2+} affect the substrate kinetic parameters. Whereas sucrose inhibits wheat germ sucrose-P synthase activity in the presence of Mg^{2+} (23), no inhibition of the spinach leaf enzyme is observed in the presence or absence of Mg^{2+}. However, Huber recently has reported that sucrose-P synthase activity in crude extracts of tobacco, peanuts, peas, and bean leaves was significantly inhibited by 50 mM sucrose, while the enzyme activity present in leaf extracts of wheat, barley, and spinach were not inhibited (13).

Of particular interest is the inhibition of the spinach leaf sucrose-P synthase by Pi. This inhibition is quite effective in the range of 5 to 10 mM. The inhibitor is competitive with UDPglucose, and it also slightly decreases the apparent affinity for fructose-6-P as the substrate $S_{0.5}$ value increases from 3.0 to 5.9 mM.

The wheat germ enzyme is rather insensitive to Pi inhibition and is not inhibited by sucrose-6-P. In the presence of 20 mM Mg^{2+}, 50% inhibition is seen with 50 mM Pi, and, in the absence of Mg^{2+}, greater than 100 mM Pi is required for 50% inhibition (22).

Sucrose-6-P, the product of the reaction, is also a competitive

inhibitor of the spinach leaf enzyme with respect to UDPglucose. However, the K_i value of 0.4 mM may be too high for the inhibition to be physiologically meaningful. Nevertheless, sucrose-P concentrations could conceivably rise, due to the possible inhibition of sucrose-P phosphatase by sucrose (8). If this occurs, then the increase of sucrose-P could feed back and inhibit sucrose-P synthase activity.

In summary, the obtained results suggest that spinach leaf sucrose-P synthase is a regulatory enzyme that is modulated by the fructose-6-P/Pi and UDPglucose/Pi ratios in the cell cytoplasm. Other mechanisms for regulating sucrose-P synthase, however, are not precluded.

The sucrose biosynthetic enzymes, UDPglucose pyrophosphorylase and sucrose-P phosphatase, are located almost exclusively in the cytosol (2, 21). Synthesis of sucrose in the leaf, therefore, would be dependent on the photosynthetic products, 3-P-glycerate and triose-P, formed in the chloroplast during photosynthesis that are able to permeate the chloroplast membrane (5, 11, 12). This translocation process is also dependent on Pi (5, 11, 12). Thus, Pi may regulate sucrose synthesis by affecting sucrose-P synthase activity and by modulating the concentration of carbon metabolites passing from the chloroplast to the cytoplasm by its translocation into the chloroplast. This would result in a decrease of cytoplasmic Pi and in an increase in the cytoplasmic carbon metabolites.

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